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## Conformational Changes of $\alpha$ -Amylase by Gamma Ray Irradiation in Aqueous Solution

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The states of amino acid residues, tryptophan, tyrosine and histidine, in  $\alpha$ -amylase were investigated using the reaction between the residues and specific chemical reagents, and the conformational degradation by irradiation was presumed from the alteration in the states of these residues or their circumstances. From the facts obtained in this work, the irradiated and inactivated enzyme protein was found to have such a conformation as more acceptable of the effect of chemical reagent than that of intact one.

In order to study the conformational changes of  $\alpha$ -amylase by gamma ray irradiation in aqueous solution, spectrophotometric titration of tyrosyl residues, optical rotatory dispersion, electrophoresis and optimum pH in enzyme reaction were investigated. These results indicated that the conformation of the enzyme was degraded and some of the tyrosyl residues embedded within a protein molecule were exposed by irradiation. The irradiated enzyme was more electronegative than the intact one and the isoelectric point of the enzyme protein was changed from pH 5.5 to pH 1.0 by the irradiation, but not changed was the optimum pH.

### INTRODUCTION

The effects of irradiation of gamma rays on  $\alpha$ -amylase in aqueous solution have been investigated and reported previously concerning the effects on the first order conformation, peptide bonds and amino acid residues of side chains in its molecule.<sup>1)</sup> From the results of such experiments as the search of small peptide removed with gel filtration, the measurement of molecular weight by ultracentrifugal analysis, the determination of N-terminal amino acid by the DNP method and the analysis of the amino acid composition, it came to the conclusion that the inactivation of  $\alpha$ -amylase by irradiation in aqueous solution was not caused by the cleavage of peptide bonds. It was suggested that the inactivation resulted from the destruction of the protein conformation or several amino acid residues essential for enzyme activity. At the point of this suggestion, the conformational degradation of  $\alpha$ -amylase by irradiation is investigated chemically in this work.

Kurihara, Shibata *et al.* have developed several reagents to differentiate between various states of amino acid residues in proteins.<sup>2~4)</sup> These reagents react with specific amino acid, and the reaction is so mild as to proceed following the states in which each amino acid residue is placed. As the conformation of protein molecule undergoes a change, the states of each amino acid residue should be changed also and therefore the reactivity of these residues with the reagent would be altered to various degrees.

The changes of the states of tryptophyl residue in  $\alpha$ -amylase by irradiation were

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studies by investigating the degree of oxidative reaction of tryptophyl residues by hydrogen peroxide in the presence of 10 percent dioxane, according to the procedure of Hachimori *et al.*<sup>4)</sup> with some modifications.

Reaction rates of tyrosyl and histidyl residues in protein molecule with diazonium-1-H-tetrazole (DHT) were compared before and after irradiation.

In this work the conformational changes of  $\alpha$ -amylase by  $\gamma$ -ray irradiation were presumed by investigating the reactivity of some amino acid residues with chemical reagent before and after the irradiation. Although the increase of the reactivity of tryptophan residue with hydrogen peroxide or of histidine and tyrosine residues with diazonium-1-H-tetrazole suggested that the irradiation caused conformational degradation on the enzyme protein, the more extensive and direct approaches seemed to be required.

Since tyrosine residue in protein molecule is considered to play an important role for the formation of stable molecular structure by its high hydrophobicity or by forming an intramolecular bond with side chain of other residues, the states of tyrosine residues in  $\alpha$ -amylase were investigated by the method of spectrophotometric titration. Helix content was estimated by measurement of optical rotatory dispersion and the changes in ionic state of enzyme protein by irradiation were investigated through the electrophoresis and optimum pH determination. The results obtained from these experiments would be described in this paper.

## MATERIALS AND METHODS

Crystalline preparation of *Bacillus subtilis*  $\alpha$ -amylase used in this work was obtained from Daiwa Kasei Co. Ltd.. When used in the spectrophotometric titration, it has undergone more purification by the filtration with Sephadex G-50 and lyophilization in order to avoid turbidity in the enzyme solution at the titration of conc alkali or acid solution. The amount of  $\alpha$ -amylase was estimated on the basis of its absorption coefficient  $E_{280m\mu}^{1\%} = 27.0$ .

Hydrogen peroxide solution was purchased from Nakarai Chemical Co. Ltd. and was used after quantitative analysis by iodine titration.

Dioxane used with hydrogen peroxide was purchased from Nakarai Chemicals, Co. Ltd. and was purified according to the established direction<sup>5)</sup> to remove the impurities of peroxides.

N-Acetyl-DL-tryptophan used as the model substance of tryptophyl residue in protein was purchased from Tokyo Kasei Co. Ltd..

DHT reagent was synthesized from 5-amino-1-H-tetrazole, purchased from Tokyo Kasei Co. Ltd., according to the method of Horinishi *et al.*,<sup>3)</sup> but the time for synthesis was 8 min. instead of 20 min. The concentration of DHT synthesized was determined by measuring the formation of N-acetylmonoazotyrosine according to the procedure of Vallee *et al.*<sup>6)</sup>

The reaction between tryptophan and hydrogen peroxide in the presence of dioxane was not so rapid in our laboratory as described by Hachimori *et al.*<sup>4)</sup> and the reactions were carried out as follows.

The reaction mixtures consisted with 1 ml of 0.25 percent  $\alpha$ -amylase solution and

2.5 ml of 1 M bicarbonate buffer, pH 8.5, 0.5 ml of various concentration of hydrogen peroxide in dioxane solution and 1 ml of distilled water were incubated at 30°C for 5 hours to complete the reaction. The concentration of dioxane in the reaction mixture was 10 percent. After refrigeration in ice water, the absorbancy of these mixtures at 282 m $\mu$  was measured using the mixtures without  $\alpha$ -amylase for cell controls. The amount of oxidized tryptophan was estimated by using the value of  $3.49 \times 10^3$  for the decrease of molecular absorption coefficient,  $\Delta \epsilon_{282\text{m}\mu}$ , of tryptophan by the oxidation.

The following procedure was employed in the reaction of  $\alpha$ -amylase with DHT. 0.2 ml of 0.135 percent  $\alpha$ -amylase solution, 2.0 ml of 1 M bicarbonate buffer, pH 8.8 and 0.8 ml of various concentration of DHT solutions were mixed and the absorbancy at 480 m $\mu$  was measured after allowing the reaction mixture to stand for 2 hours at room temperature.

Colorimetric measurements were made with an EPU-2, Hitachi photoelectric spectrophotometer and absorption spectrum was drawn with an EPS-2, Hitachi automatic recording spectrophotometer.

Titration was carried out in 0.5 M KCl solution in order to diminish the effect of the changes in ionic strength. A 3 N-KOH solution and 6 N HCl were titrated into 16 ml of  $1.2 \times 10^{-5}$  M  $\alpha$ -amylase in 0.5 M KCl solution with a ultramicrobiuret for the forward titration and the reverse titration respectively. Immediately after the pH measurement with a Hitachi Horiba M4 pH meter, differential absorbance at 297 m $\mu$  was measured using  $1.2 \times 10^{-5}$  M  $\alpha$ -amylase solution containing 0.5 M KCl for a cell control with an EPU-2, Hitachi photoelectric spectrophotometer. The value obtained by this measurement was corrected with the volume increased by that alkali and acid titration.

Optical rotatory dispersion was measured by a Yanagimoto O. R. D. Apparatus using a 5 cm cell at constant temperature, 20°C, in a range of 310–550 m $\mu$ . The concentration of  $\alpha$ -amylase in this measurement was 0.067 percent and the solvent was distilled water.

Electrophoresis was carried out by two methods. One is the moving boundary method using a Tiselius electrophoresis apparatus and the other is the zone electrophoresis method using cellulose acetate membrane as a supporter.  $\alpha$ -Amylase used in the Tiselius method was prepared by dissolving in the buffer of various pH, ionic strength  $\mu=0.1$ , and by dialysis for 36 hours at 5°C against the same buffer. Electrophoresis was carried out using a Hitachi HT-B Tiselius apparatus at 4°C sending an electric current of 15 mA.  $\alpha$ -Amylase used in the zone electrophoresis method was purified by the same method in the experiment of tyrosine titration. An 0.0008 ml of 0.1 percent  $\alpha$ -amylase solution in distilled water was putted on a cellulose acetate membrane bufferized in the 0.07 M Na veronal buffer, pH 8.95, and the electrophoresis was carried out for 40 min at room temperature in the same buffer, sending an electric current of 0.8 mA per 1 cm of the membrane width.

Activity measurement of  $\alpha$ -amylase was made according to the Nelson's method<sup>7)</sup> where the amount of aldehyde groups of hydrocarbons in the enzyme reaction mixture was measured colorimetrically. Details were described in the previous paper.<sup>8)</sup>

Gamma ray irradiations were carried out using a Toshiba RE 1010 <sup>60</sup>Co (100 Ci) Irradiation Facility with a dose rate of  $4.63 \times 10^4$  rad per hour<sup>9)</sup> and a 2000 Ci <sup>60</sup>Co

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Irradiation Facility with the dose rate of  $1.2 \times 10^5$  rad per hour in the Institute for Chemical Research, Kyoto University. Before irradiation,  $\alpha$ -amylase was dissolved into twice distilled water in a concentration of 0.25 percent for reaction with hydrogen peroxide and of 0.135 percent for that with DHT.

### RESULTS

#### States of tryptophyl residues in intact $\alpha$ -amylase and irradiated one

It was reported by Hachimori *et al.* that the oxidation of N-acetyl-tryptophan and tryptophyl residues in protein molecule with hydrogen peroxide in the presence of 10 percent dioxane was completed at 2–4°C for 30 minutes and 2.5 hours respectively, but in our laboratory it took further incubation at 30°C of 3 hours for N-acetyl-DL-tryptophan solution and of 5 hours for  $\alpha$ -amylase solution to obtain a constant value of absorbancy at 282 m $\mu$  as shown in Fig. 1. Although the reason for the difference

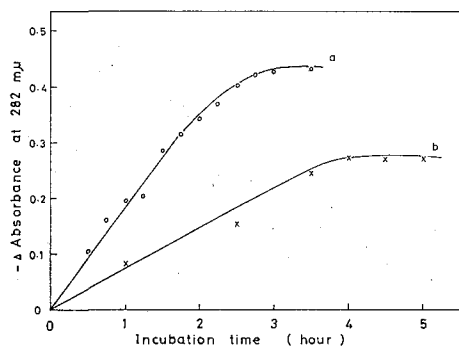


Fig. 1. The degree of oxidation after various time of incubation at 30°C. Curve a indicates the oxidation of 0.15 mM N-acetyl-DL-tryptophan with 20 mM  $\text{H}_2\text{O}_2$ . Curve b indicates the oxidation of 0.05 percent  $\alpha$ -amylase with 40 mM  $\text{H}_2\text{O}_2$ .

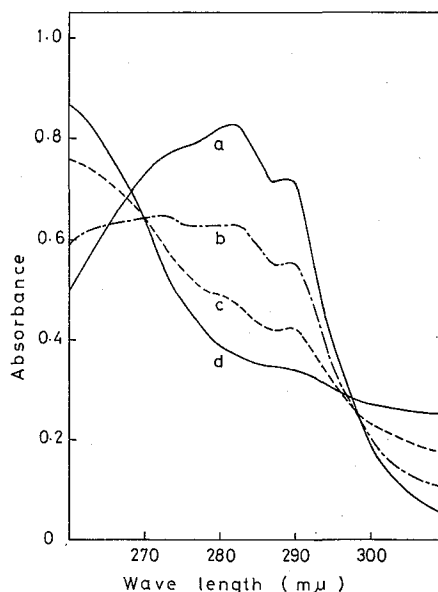


Fig. 2. The spectral change of 0.15 mM N-acetyl-DL-tryptophan by treatment with  $\text{H}_2\text{O}_2$  at 30°C for 2.5 hours. The concentration of  $\text{H}_2\text{O}_2$  are none for curve a, 2 mM for curve b, 10 mM for curve c and 20 mM for curve d respectively.

in reaction rate was indistinct, it may be resulted from any peroxides remained even after purification. At any rate, the ultraviolet absorption spectra of N-acetyl-DL-tryptophan oxidated by the so long incubation with hydrogen peroxide in dioxane seemed to be fairly similar to that of Hachimori *et al.*'s data in both the shape and the two isobestic points observed at 270 and 295 m $\mu$ . The spectrum of N-acetyl-DL-tryptophan solution and that of oxidized one were shown in Fig. 2. The incubation

temperature for oxidation in this experiment was 30°C. As the temperature was set higher than 40°C, the two isobestic points disappeared. Therefore, the incubation was carried out at 30°C for 5 hours as described in METHODS. The reduction of absorbancy at 282 m $\mu$ , which was obtained after the incubation of  $\alpha$ -amylase with hydrogen peroxide in dioxane, was plotted against H<sub>2</sub>O<sub>2</sub> concentration. Figure 3 shows the processes

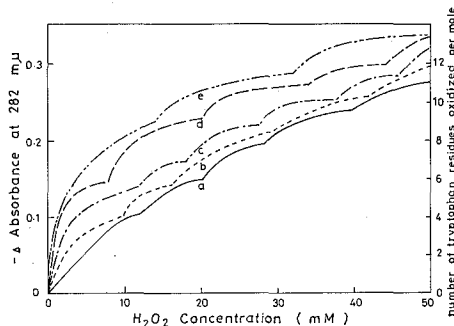


Fig. 3. The processes of oxidation of tryptophen residues in intact and irradiated  $\alpha$ -amylase.

The concentration of  $\alpha$ -amylase is  $0.69 \times 10^{-5}$  M.

The irradiation dose and the survival ratio of enzyme activity are: curve a, none 100 %, b,  $1.5 \times 10^5$  rad 80 %, c,  $2.9 \times 10^5$  rad 56 %, d,  $4.4 \times 10^5$  rad 46 %, e,  $13.5 \times 10^5$  rad 0 %.

of oxidation of tryptophan residues in intact and irradiated  $\alpha$ -amylase. According as the dose irradiated increasing and inactivation of enzyme proceeds, the number of tryptophan residues oxidized with low concentration of hydrogen peroxide is found to be increased. The oxidation process of  $\alpha$ -amylase which was not irradiated, but treated with alkali at pH 13.0 for 2 hours, was almost similar to that of enzyme irradiated with  $4.4 \times 10^5$  rad and inactivated to 46 percent of its original activity.

#### States of histidyl and tyrosyl residues in intact and irradiated $\alpha$ -amylase

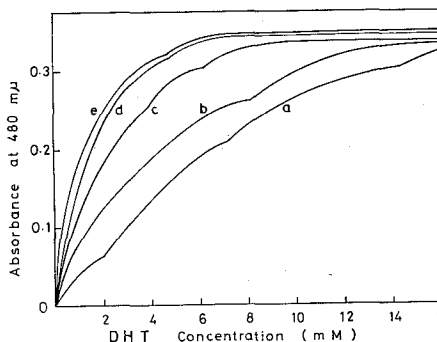


Fig. 4. The reaction curves of B. s.  $\alpha$ -amylase with DHT.

The concentration of  $\alpha$ -amylase in reaction mixtures was  $1.2 \times 10^{-6}$  M.

a: intact, b: irradiated by  $3.7 \times 10^4$  rad and remaining 80 percent of original, c: irradiated by  $7.4 \times 10^4$  rad and remaining 54 percent of original activity, d: irradiated by  $14.7 \times 10^4$  rad and remaining 20 percent of original activity, e: irradiated by  $29.4 \times 10^4$  rad and remaining 2 percent of original activity.

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DHT had been proposed by Horinishi *et al.*<sup>3)</sup> as a reagent to differentiate various states of histidine residues in protein. The amount of bisazo-histidine produced in the reaction between DHT and proteins was determined by measuring the absorbancy at 480  $m\mu$ . Afterward, 1966, Vallee *et al.*<sup>6)</sup> have found that the azoderivatives of tyrosine also show considerable absorbance in the range from 400 to 600  $m\mu$ . At pH 8.8, the absorption maximum of monoazotyrosine and bisazotyrosine are at 480  $m\mu$  with  $\epsilon$  value of  $5.2 \times 10^3$  and at 550  $m\mu$  with  $\epsilon$  value of  $1.41 \times 10^4$  respectively and these spectra accumulate on that of bisazohistidine. In the case of  $\alpha$ -amylase, the reaction between the enzyme protein and DHT progressed as shown in Fig. 4, so far as measuring the amount of azoderivatives by the absorbancy at 480  $m\mu$  at pH 8.8. Curve a is that of intact  $\alpha$ -amylase and b, c, d and e are that of irradiated one. The reactivity of  $\alpha$ -amylase with DHT was found to be extremely increased by irradiation. As the absorbance measured at 480  $m\mu$ , however, had much possibility to be the sum of the various azoderivatives of amino acids as described above, the absorption spec-

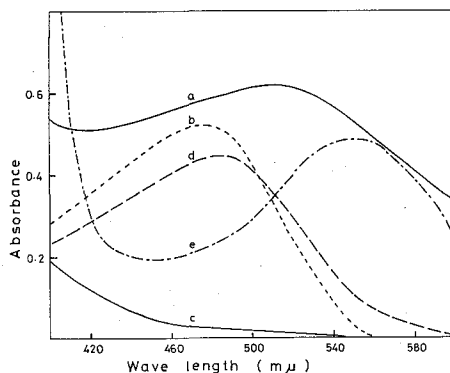


Fig. 5. Absorption spectra of  $\alpha$ -amylase, tyrosine and histidine treated with DHT at pH 8.8.

a)  $1.2 \times 10^{-6}$  M  $\alpha$ -amylase treated with 14 mM DHT. b)\* bisazo-N-acetylhistidine obtained by treating  $5 \times 10^{-3}$  M N-acetyl histidine with  $1.5 \times 10^{-2}$  M DHT. c)\* monoazo-N-acetylhistidine obtained by treating  $5 \times 10^{-3}$  M N-acetylhistidine with  $6 \times 10^{-3}$  M DHT. d) monoazo-N-acetyltyrosine ethyl ester obtained by treating  $8 \times 10^{-4}$  M N-acetyl tyrosine ethyl ester with  $8.9 \times 10^{-5}$  M DHT. e) bisazo-N-acetyltyrosine ethyl ester obtained by treating  $3.3 \times 10^{-5}$  M N-ATEE with  $4.4 \times 10^{-2}$  M DHT.

Table 1. Reduction of amino acid in  $\alpha$ -amylase by treating with various concentration of DHT.  $\alpha$ -Amylase treated with DHT was submitted to amino acid analysis after filtration through the Sephadex G-25 column.

Results are expressed as moles of amino acid per mole of enzyme and are calculated on the basis of 39 glycines/mole.

Mole ratio of DHT to $\alpha$ -amylase Amino acid	None moles/mole of enz.	500		1,000		4,500	
		mole	-- $\Delta$ mole	mole	-- $\Delta$ mole	mole	-- $\Delta$ mole
Tyrosine	24	16.5	7.5	7.5	16.5	4.1	20.0
Lysine	26	3.5	22.5	1.5	24.5	1.3	24.7
Histidine	13	9.0	4.0	5.7	7.3	6.1	6.9

\* b) and c) are the data of Vallee *et al.*<sup>6)</sup>

trum and amino acid composition of  $\alpha$ -amylase treated with DHT were investigated. The spectrum was shown in Fig. 5 together with the azoderivatives of tyrosine and histidine. It showed a broad curve with a maximum peak at 500  $m\mu$  and was presumed the accumulation of other spectra, curve b, c and d. On the other hand, the amino acid composition of  $\alpha$ -amylase was measured by the method of Stein and Moore<sup>10</sup> before and after the treatment with DHT. The amount of amino acid residues was not so changed without lysine, tyrosine and histidine whose amounts were reduced considerably as shown in Table 1.

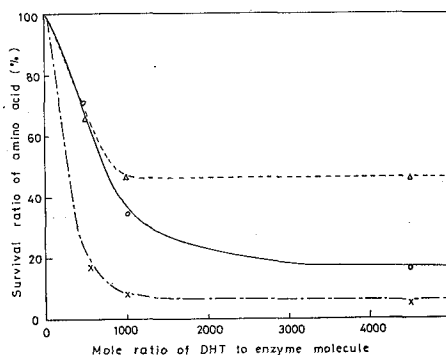


Fig. 6. Survival ratio of amino acid after treating with DHT.  
(●) histidine, (○) tyrosine, (△) lysine.

The ratio of moles modified to that of intact was shown in Fig. 6. Excepting lysine which was modified most rapidly, but not interfere spectrally, both the reactions of tyrosine and histidine with DHT progressed almost similarly to each other at least in the low molar ratio of DHT to  $\alpha$ -amylase.

#### Spectrophotometric titration of tyrosine residues

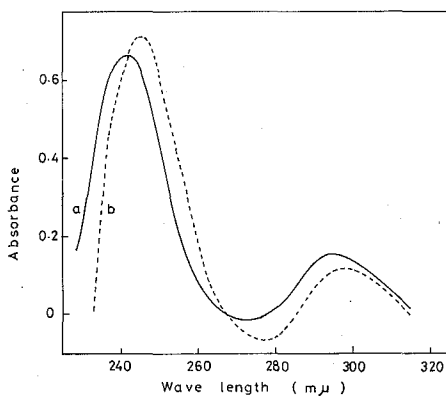


Fig. 7. U. V. difference spectra of L-tyrosine (a) and  $\alpha$ -amylase (b) in alkaline solution versus in neutral solution. Both L-tyrosine and  $\alpha$ -amylase in 0.5 M KCl, 0.5 M KOH solution, pH 13.0 were incubated for 3 hours at room temperature and then the absorbancy was measured against the solution in 0.02 M tris buffer containing 0.5 M KCl, pH 7.49. The spectral determinations were carried out at a concentration of  $6 \times 10^{-5}$  M for L-tyrosine and of  $3.2 \times 10^{-6}$  M for  $\alpha$ -amylase.



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The phenolic hydroxyl group is ionized in alkaline solution and its absorption spectrum shifts to longer wave length region as a phenoxide ion. Difference spectra of L-tyrosine and  $\alpha$ -amylase in alkaline solution versus in neutral solution were shown in Fig. 7. Two maximum peaks were observed in both spectra, at 242 and 295  $m\mu$  for L-tyrosine, at 245 and 297  $m\mu$  for  $\alpha$ -amylase. From curve a in Fig. 7, a  $\Delta\epsilon_{245m\mu}$ , the difference of molecular absorption coefficient at 245  $m\mu$ , of  $1.106 \times 10^4$  and a  $\Delta\epsilon_{297m\mu}$  of  $2.377 \times 10^3$  were obtained. These values are in good agreement with those reported previously<sup>11,12)</sup> and the number of ionized tyrosine residue in protein was estimated using the latter value. An alkaline titration curve and back titration curve in intact

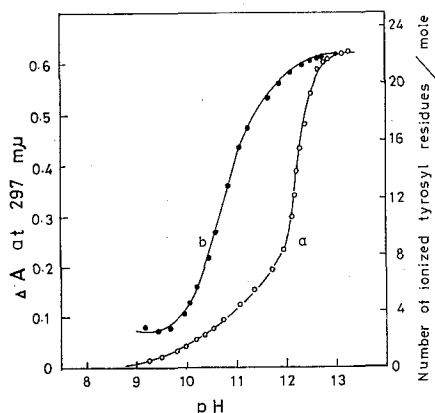


Fig. 8. Spectrophotometric titration curve of intact  $\alpha$ -amylase ( $1.18 \times 10^{-5}$  M). Curve a, forward titration curve b, reverse titration, reversed after exposure to pH 13.0 for 1 hour.

$\alpha$ -amylase were shown in Fig. 8. Although at pH 12, only 8 or 9 moles of tyrosine were ionized, the ionization progressed suddenly beyond the pH and as the result about 24 moles of tyrosine per mole were titrated at pH 13.0 finally. The value, 24 moles of tyrosine is in good agreement with the value obtained by the amino acid composition analysis.<sup>1)</sup> Reverse titration carried out after 1 hour incubation at pH 13.0 showed

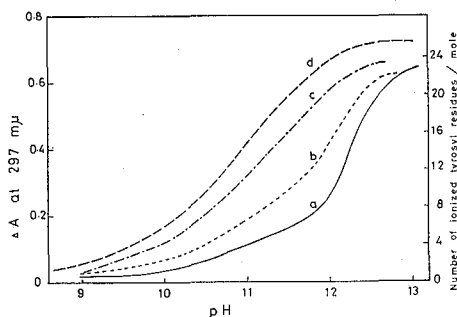


Fig. 9. Spectrophotometric titration curves of intact and irradiated  $\alpha$ -amylase. Concentration of enzyme solution was  $1.18 \times 10^{-5}$  M. Irradiation dose and survival activity ratio were, curve a; none, 100 %, b;  $7.4 \times 10^4$  rad, 60 %, c;  $14.8 \times 10^4$  rad, 31 %, d;  $29.6 \times 10^4$  rad 4 %.

that the more tyrosine residues were ionized than that in forward titration at the same pH value because of the alkaline denaturation and conformational degradation.

Spectrophotometric titration was applied also in the enzyme irradiated with various dose of gamma rays. As shown in Fig. 9, with the increase of the irradiated dose and inactivation rate the ionization was found to proceed at lower pH value than in intact enzyme protein.

In order to investigate the states of tyrosine residues more precisely and to calculate the pK value for ionization these data were treated according to the following equation proposed by Hermans *et al.* and developed by Murachi *et al.*<sup>(1)</sup>

$$[H^+] \cdot \Delta\epsilon = \Delta\epsilon_{\max} \cdot K - \Delta\epsilon \cdot K \quad (1)$$

where  $\Delta\epsilon$  is a difference in molar absorptancy at the pH value,  $\Delta\epsilon_{\max}$  is a difference in molar absorptancy attainable after a complete ionization and  $K$  is the apparent dissociation constant. From the intercept on the abscissa and the slope of the straight line obtained by a plotting of  $[H^+] \cdot \Delta\epsilon$  versus  $\Delta\epsilon$ , a  $\Delta\epsilon_{\max}$  value and a pK (app) value could be calculated. In the case of this  $\alpha$ -amylase, however, this plotting gave a curve instead of a line because of the tyrosine as much as 24 moles per mole of enzyme protein, which were in various state in the protein molecule. It is impossible to calculate the pK value of each tyrosine residue considering the experimental error, but it seems reasonable to consider that several residues placed in the state similar to each other in protein molecule would be ionized as a group with a pK value. Twenty four moles of tyrosine residues were divided into 4 groups expediently and the number of

Table 2. The number and pK value of tyrosine residues ionized. These values were obtained by the plotting according to the equation (1).

Activity	Dose ( $\times 10^{-4}$ rad)	0 100%	7.4 60%	14.8 31%	29.6 4%
A	n	2.1	4.2	7.5	10.7
	pK	9.76	9.80	9.82	9.86
B	n	2.8	4.8	9.0	3.2
	pK	10.44	10.56	10.62	10.43
C	n	4.5	3.7	7.1	11.1
	pK	11.28	10.96	11.0	10.85
D	n	16.1	12.8	—	—
	pK	12.16	11.82	—	—
n(A+B+C+D)		25.5	25.5	23.6	25.0

tyrosine ionized and the pK value were calculated, as shown in Table 2. With the decrease of the enzyme activity by irradiation, the number of residues ionized with lower pK value was increased. This fact indicates that the tyrosines buried within the protein molecule had come to be exposed by irradiation.

#### Optical rotatory dispersion

Presuming that the conformational degradation is accompanied with the decrease

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in the amount of  $\alpha$ -helix in protein molecule, helical content of intact and irradiated  $\alpha$ -amylase were estimated by the measuring of optical rotatory dispersion.

Specific rotation at  $500\text{ m}\mu$  at  $20^\circ\text{C}$ ,  $[\alpha]_{500}^{20}$ , optical rotatory dispersion constant,  $\lambda_c$ , excess R-handed helical content,  $x^H$  and  $\beta$ -structural content,  $x^\beta$  were listed in Table 3.  $\lambda_c$  was determined by the method of Lowry's plotting, and the values,  $x^H b_0^H$  and  $a_0$ , were obtained by the method of Moffitt's plotting according to the Moffitt equation

Table 3. Optical rotatory dispersion of intact and irradiated  $\alpha$ -amylase.

$[\alpha]_{500}^{20}$  is the specific rotation at  $500\text{ m}\mu$ ,  $20^\circ\text{C}$ .

$\lambda_c$  is optical dispersion constant.

$x^H b_0^H$ ,  $a_0$  and  $x^\beta a_0^\beta$  are the terms in the equation (2) and (3).

$x^H$  and  $x^\beta$  show the content of  $\alpha$ -helix and of  $\beta$ -structure respectively and had been calculated by assuming  $\lambda_0 = 212\text{ m}\mu$ ,  $b_0^H = -630$ ,  $a_0^R = -560$ ,  $a_0^H = +680$  and  $a_0^\beta = +800$ .

Dose irradiated	None	$0.5 \times 10^5\text{ rad}$	$3.7 \times 10^5\text{ rad}$
Enzyme Activity	100 %	15 %	0
$[\alpha]_{500}^{20}$ (degree)	- 59.7	- 67.4	- 88.3
$\lambda_c$ (m $\mu$ )	266	252	228
$x^H b_0^H$ (degree)	-234	-180	- 90
$x^H$	0.37	0.28	0.14
$a_0$ (degree)	-215	-240	-375
$x^\beta a_0^\beta$ (degree)	87	122	86
$x^\beta$	0.109	0.152	0.107

$$[M] = \frac{a_0 \lambda_0^2}{\lambda^2 - \lambda_0^2} + \frac{x^H b_0^H \lambda_0^4}{(\lambda^2 - \lambda_0^2)^2} \quad (2)$$

$$a_0 = a_0^R + x^H a_0^H + x^\beta a_0^\beta. \quad (3)$$

By the irradiation,  $-[\alpha]_{500}^{20}$  was increased, but  $\lambda_c$  and  $x^H$  were decreased. The

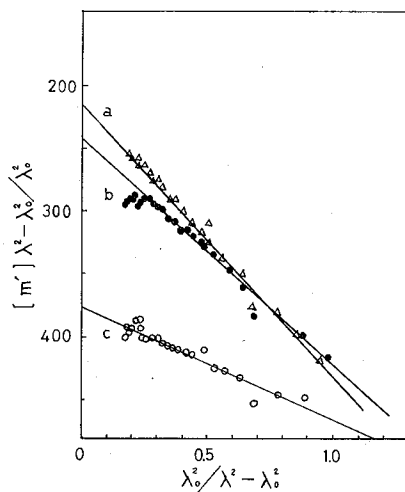


Fig. 10. Moffitt's plotting of the results of optical rotatory dispersion measurements on non-irradiated and irradiated  $\alpha$ -amylase in aqueous solution.

results were shown in Fig. 10. From these results the amount of  $\alpha$ -helix in protein molecule was seemed to be decreased by the irradiation.

### Electrophoresis

In order to investigate the effect of irradiation on the ionic state of  $\alpha$ -amylase, electrophoresis was carried out before and after the irradiation.

Electrophoretic mobilities of intact and irradiated  $\alpha$ -amylase with  $8.3 \times 10^4$  rad were measured in various pH solution using a Tiselius type apparatus. The results were shown in Fig. 11. As  $\alpha$ -amylase is precipitated in lower pH region, the measurements were made in the higher pH region than 6.8 and the isoelectric point was obtained by the extrapolation of the mobility to zero. The isoelectric point of intact  $\alpha$ -amylase is at pH 5.5, but that of irradiated  $\alpha$ -amylase seems to be at pH 1.0 and the electrophoretic mobility in negative was found to be increased by irradiation.

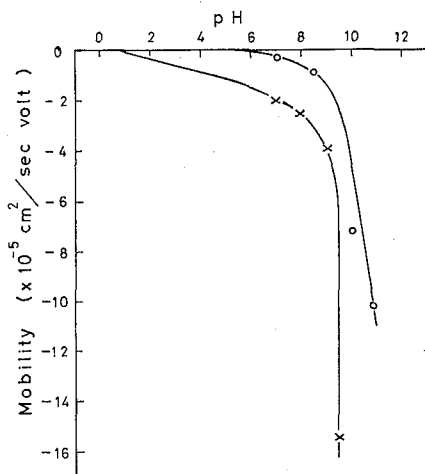


Fig. 11. Electrophoretic mobility of intact and irradiated  $\alpha$ -amylase.

( $\times$ ) is intact  $\alpha$ -amylase. ( $\circ$ ) is  $\alpha$ -amylase irradiated by  $8.3 \times 10^4$  rad.

Buffers used in this experiment were phosphate buffer for pH 6.8–7, veronal-HCl buffer for pH 8–9 and glycine-NaOH buffer for pH 9.7–10.7 respectively, but the ionic strength was adjusted in 0.1 in all the cases.

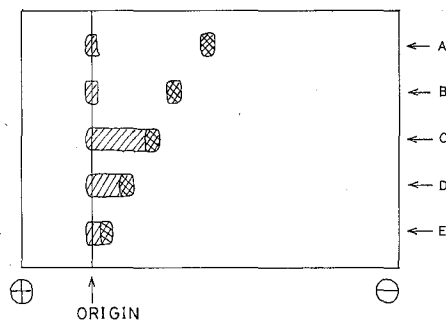


Fig. 12. Electrophoretic pattern of intact and irradiated  $\alpha$ -amylase on a cellulose acetate membrane. The irradiated dose and the remaining activity ratio of each patterns are,

- A ; 0, 100 %
- B ;  $3.08 \times 10^4$  rad, 67 %
- C ;  $6.16 \times 10^4$  rad, 36 %
- D ;  $6.16 \times 10^4$  rad, 4 %
- E ;  $12.32 \times 10^4$  rad, 3 %.

The electrophoretic patterns on a cellulose acetate membrane were shown in Fig. 12. It was found that the mobility to the negative electrode was decreased with the inactivation by irradiation.

### Optimum pH in enzyme reaction

The optimum pH in enzyme reaction was measured in order to investigate whether the ionic state was changed by irradiation or not, because the rate of enzymatic reac-

# Conformational Changes of $\alpha$ -Amylase by Gamma-irradiation

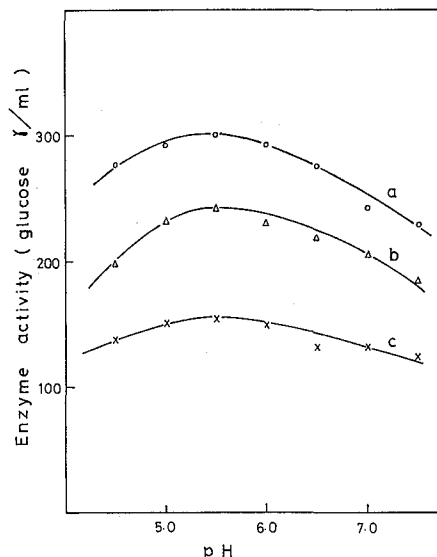


Fig. 13. Activity curve versus pH values of intact and irradiated  $\alpha$ -amylase. Buffer used in this measurement was 0.1 M acetate sodium acetate buffer, pH 4.5-7.5. a ; non irradiated, b ; irradiated by  $6.5 \times 10^4$  rad, c ; irradiated by  $9.8 \times 10^4$  rad.

tion depends on the ionic state of enzyme molecule. The results were shown in Fig. 13. The activity curves versus pH values of intact and irradiated enzyme were essentially identically and the optimum pH was 5.5 in every case.

## DISCUSSION

It can be said very simple method to presume the state of amino acid residues in protein molecule by the reactivity of residues with a chemical reagent, when the amount of reaction product can be measured colorimetrically. The method, however, seems to be defective in two points. One is that such a specific chemical reagent as react with only one species of amino acid is very rare and one is that it is impossible to check whether or not the protein has accepted the changes in its conformation by the modification of residues with that reagent. Therefore the changes in the reactivity with a reagent may show rather the difference in the modification effect to the protein conformation than the actual changes in its conformation.

It would be reasonable, however, to consider that changes in the sensitivity to the reagent effect show conformational change of a kind, and modifications of  $\alpha$ -amylase with two species of chemical reagent were carried out. Either oxidation of tryptophan by hydrogen peroxide in dioxane solution or azo coupling of tyrosine and histidine by DHT reagent in irradiated  $\alpha$ -amylase was found to proceed in lower concentration of reagent than intact enzyme protein. Intact  $\alpha$ -amylase, 4 of 14 moles tryptophan residue were oxidized in the low concentration of hydrogen peroxide and they seems to be exposed at the surface of the enzyme molecule. Following the increase of  $H_2O_2$ , the oxidation progresses through several steps, but even at the maximum concentration of  $H_2O_2$ , 50 mM, in this experiment three moles of tryptophan were failed in oxi-

dation.

In the irradiated  $\alpha$ -amylase, almost all tryptophan residues were oxidized by this concentration of  $H_2O_2$ .

In the coupling reaction of  $\alpha$ -amylase with DHT, such steps as shown in the oxidative reaction of tryptophan were not found, since the absorbance at  $480 m\mu$  resulted from various azoderivatives, monoazotyrosine, bisazotyrosine and bisazohistidine. The reaction progress of DHT with amino acid residues in protein are in variety with the species of that protein. For example, in the case of carboxypeptidase,<sup>13)</sup> tyrosine was more reactive than histidine and lysine at the low molar ratio of DHT to protein molecule. On the other hand, in the case of RNase lysine and histidine were diazotized initially and then tyrosine was reacted. But in the case of  $\alpha$ -amylase, tyrosine and histidine both of which are absorptive in the region of  $400-600 m\mu$  react with DHT almost similarly at least in the low concentration of the reagent. This fact makes impossible to determine the amount of coupled tyrosine and histidine with DHT and to differentiate the states of these residues separately in the case of  $\alpha$ -amylase. Therefore the curves in Fig. 4 show the reaction progresses of both tyrosine and histidine with DHT accumulatively. From the tendency of these residues to react with chemical reagent more easily by irradiation, it could not be concluded directly that the secondary or tertiary structure of this enzyme protein had been changed by irradiation for the reason described above, but that the enzyme protein had become more sensitive to the effect of chemical reagent by irradiation.

Tyrosine is considered to have high hydrophobicity in the non ionized state and tyrosine residues in protein molecule could be divided roughly into two groups, one is exposed in the surface of molecule surrounded with solvent and the other is buried in the interior of molecule constituting the hydrophobic region. In the case of intact B. s.  $\alpha$ -amylase, exposed tyrosine which is titrated with the same dissociation constant as tyrosine solution,  $pK=9.8$ , was only 2 moles among the 24 moles of tyrosine residue in this protein. The majority of tyrosine residues, about 16 moles, was ionized only after the pH of this enzyme solution was raised beyond 12 and the protein conformation was degraded by a denaturation. It should be reasonable to consider that this enzyme has a considerably rigid structure in its native state.

In the irradiated  $\alpha$ -amylase, however, the amount of tyrosine residues ionized in lower pH region was increased. This fact suggests that the irradiation causes the conformational degradation resulted in the exposure of buried tyrosine or the conformational changes into the more sensitive one to the alkali denaturation. As the measurement of optical rotatory dispersion shows the decrease of helix content in this protein molecule by irradiation, it would be more reasonable to consider the conformational degradation accompanied with the unfoldings of  $\alpha$ -helix for the effect of irradiation rather than the changes only in the sensitivity to alkali or other chemical reagents such as hydrogen peroxide, diazonium-1-H-tetrazole, as reported previously.<sup>1)</sup> Although the helical content was decreased lower than a half by the irradiation of  $3.7 \times 10^5$  rad, the dose is three times as much as the dose required for the complete inactivation of the enzyme. The decrease of helical content found in the  $0.5 \times 10^5$  rad irradiated  $\alpha$ -amylase remaining 15 percent of its original activity was not so large

(from 37 % to 28 %), that it cannot be concluded now at least that the conformational degradation causes the enzyme inactivation directly. But the decrease of the helical content, about 10 percent, may be resulted in the conformational degradation of active sites in enzyme which makes impossible to exhibit the activity.

Furthermore, considering the exposure by irradiation of buried tyrosine residues which would have played an important role for maintenance of the rigid structure, the conformation may have been degraded more intensely than the degree supposed from the reduction of helical content.

From the results of the electrophoretic experiment, irradiated  $\alpha$ -amylase was found to be more electronegative than intact one. Although the apparent electronegativity suggests the two phenomena, one the reduction of positive charge and the other the increase of negative charge, such facts as the liberation of ammonia found in the analysis of free ammonia,<sup>1)</sup> the reduction of basic amino acid residues, such as lysine, histidine and arginine, found in the analysis of amino acid composition<sup>1)</sup> and the flawlessness in the peptide chain in the irradiated  $\alpha$ -amylase indicate the former to occur by the irradiation. In spite of the changes in the electrophoretic mobility and in the isoelectric point of  $\alpha$ -amylase by irradiation, the optimum pH of irradiated enzyme was similar to that of intact one. Homogeneity in the electrophoretic pattern indicates that all of the enzyme molecule exposed to irradiation was accepted changes similarly at least electrophoretically. It seems likely that the changes of the conformation or of the constituent amino acid residues in the active site of this enzyme cause the complete inactivation, so that the optimum pH appeared in the enzyme reaction is not changed. This consideration is in good agreement with the "all or none" theory proposed in the work of kinetics.

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